

# EXHIBIT 11



# Mosquito-fungus interactions and antifungal immunity

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## ABSTRACT

The mosquito immune system has evolved in the presence of continuous encounters with fungi that range from food to foes. Herein, we review the field of mosquito-fungal interactions, providing an overview of current knowledge and topics of interest. Mosquitoes encounter fungi in their aquatic and terrestrial habitats. Mosquito larvae are exposed to fungi on plant detritus, within the water column, and at the water surface. Adult mosquitoes are exposed to fungi during indoor and outdoor resting, blood and sugar feeding, mating, and oviposition. Fungi enter the mosquito body through different routes, including ingestion and through active or passive breaches in the cuticle. Oral uptake of fungi can be beneficial to mosquitoes, as yeasts hold nutritional value and support larval development. However, ingestion of or surface contact with fungal entomopathogens leads to colonization of the mosquito with often lethal consequences to the host. The mosquito immune system recognizes fungi and mounts cellular and humoral immune responses in the hemocoel, and possibly epithelial immune responses in the gut. These responses are regulated transcriptionally through multiple signal transduction pathways. Proteolytic protease cascades provide additional regulation of antifungal immunity. Together, these immune responses provide an efficient barrier to fungal infections, which need to be overcome by entomopathogens. Therefore, fungi constitute an excellent tool to examine the molecular underpinnings of mosquito immunity and to identify novel antifungal peptides. In addition, recent advances in mycobiome analyses can now be used to examine the contribution of fungi to various mosquito traits, including vector competence.

## 1. Introduction

Fungi hold nutritional value for larval and adult mosquitoes, and thus are mosquito prey (Asahina, 1964). Fungal commensals can form longer-term associations in the mosquito larval gut, with little to no impact on host survival (Tuzet and Manier, 1947). In contrast, some water molds (Chromista) and fungi utilize mosquitoes as their growth medium, most often with detrimental or lethal effects on their host (Braun, 1855; Galli-Valerio and Rochaz de Jongh, 1906; Eckstein, 1922; Roubaud and Tomanoff, 1930; Couch, 1935). The potential use of such fungal entomopathogens to control mosquito populations has largely driven the research on fungal-mosquito interactions over more than a century (reviewed in e.g. Christophers, 1952; Castillo and Roberts, 1980a,b; Jenkins, 1964; Roberts, 1974; Lakon, 1919; Kanzok and Jacobs-Lorena, 2006; Scholte, 2005). While this holds true today (e.g. Lovett et al., 2019), mosquito microbiome studies have recently been extended to fungi (Kaufman et al., 2008; Tajedin et al., 2009; Muturi et al., 2016; Luis et al., 2019). These studies provide new insight into the range of mosquito-fungi encounters that may be fleeting, but may stimulate mosquito immunity through fungal surface molecules and/or

secondary metabolites.

Entomopathogenic chromista and fungi infect mosquitoes through different routes, including active penetration of the body wall, through wounds, or through ingestion, dependent on the pathogen as well as the life stage of the mosquito (e.g. Clark et al., 1966, 1968; McCray et al., 1973; Greenfield et al., 2014). Most commonly, entomopathogens isolated from field-caught mosquitoes belong to the genus *Coelomomyces*, which are ascomycetes fungi in the order of Blastocladales (Castillo and Roberts, 1980a,b; Couch and Umphlett, 1963). *Coelomomyces* fungi infect mosquito larvae through their aquatic zoospores. Zoospores attach to the larval intersegmental cuticle, and penetrate the host body wall by a penetration tube. Hyphal growth and sporangia formation occurs in the hemolymph, leading to larval death (reviewed in Araujo and Hughes, 2014; Scholte et al., 2004). However, their obligate two-host life cycle limits their use in mosquito control programs (Laird et al., 1992; Service, 1977). Currently, entomopathogens being proposed for mosquito control are ascomycetes fungi in the genera of *Beauveria* and *Metarhizium* in the order Hypocreales; Farenhorst et al. (2011); Lovett et al. (2019); Popko et al. (2018); Scholte et al. (2005). In general, fungi in these two genera infect mosquitoes via asexual

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spores called conidia, which germinate and produce penetration pegs that breach the host cuticle. Upon reaching the hemolymph, the fungus proliferates and forms yeast-like blastospores that rapidly colonize the host leading to its death (reviewed in Scholte et al., 2004).

Recognition of largely unknown fungal and water mold-derived molecules by mosquito pathogen recognition receptors (PRRs), activates mosquito epithelial, cellular and humoral immune responses. Mosquito cellular immune responses include phagocytosis, encapsulation, and nodulation (Bartholomay and Michel, 2018; Hillyer and Strand, 2014). Humoral immune responses include complement-like reactions, melanization, and the expression of antimicrobial peptides (Blandin et al., 2008; Nakhleh et al., 2017; Rhodes and Michel, 2017). These immune responses are regulated through proteolytic activation of key immune factors and/or at the transcriptional level by several signal transduction pathways including Toll pathway, the immune deficiency (IMD) pathway, Janus Kinase (JAK)-signal transducer and activator of transcription (STAT), and Jun N-terminal kinase (JNK) and mitogen-activated protein kinase (MAPK) p38 pathway (Nakhleh et al., 2017; Rhodes and Michel, 2017). The most commonly observed immune response against fungi and water molds is melanization (Brey et al., 1988; Coluzzi, 1966; Mc Innis and Zattau, 1982; Clark et al., 1968). Molecular insights of mosquito antifungal immune responses are driven by comparative invertebrate immunology (Al Souhail et al., 2016; Chen et al., 2010; Valanne et al., 2011), and experiments performed using a small number of fungal laboratory infection models (Vizioli et al., 2001a; Ramirez et al., 2019; Rhodes et al., 2018; Yassine et al., 2012).

This review will summarize the potential environmental and molecular interactions of mosquito adults and larvae with fungi and water molds. The first two sections will provide an overview of their reported environmental encounters, and detail the potential routes of infection. The sum of these interactions across the symbiotic continuum shapes the mosquito antifungal immune responses, which we describe in the final section of this review.

## 2. Mosquito-fungus encounters in the environment

Fungi are ubiquitous and found in all mosquito habitats (Goh and Hyde, 1996). In addition, the aquatic environment of mosquito larvae provides habitat for water molds. This close proximity leads to a myriad

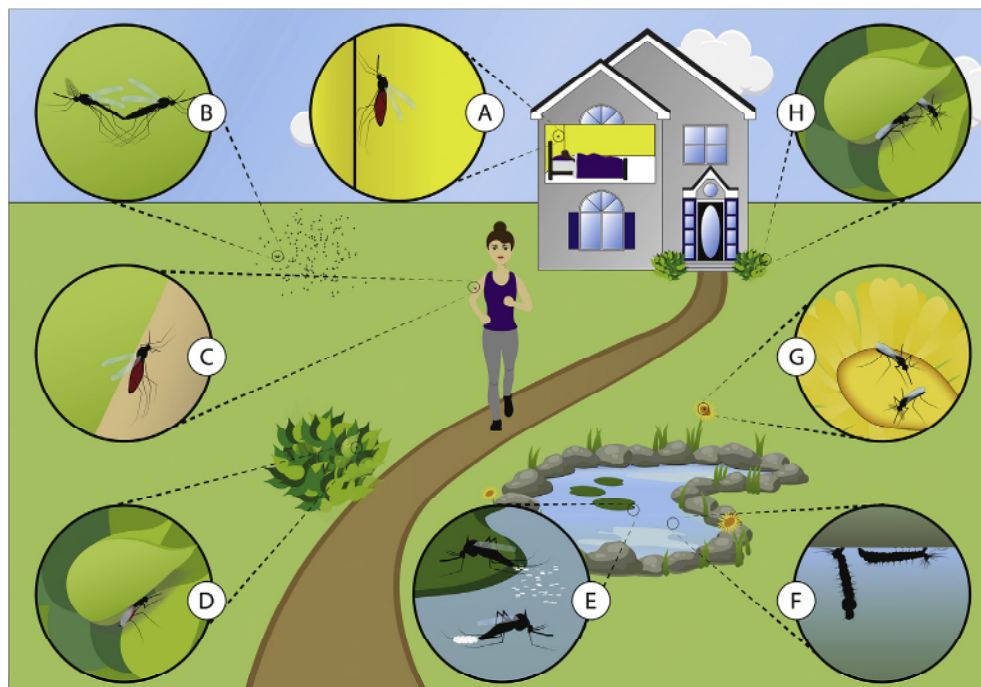
of possible interactions between mosquitoes and fungi as well as water molds throughout the mosquito's life history (Fig. 1). We mined the existing literature (see Supplementary Text for methods and references) and found records for 158 and 43 species of fungi and water molds, respectively, having been observed in/isolated across 149 mosquito species (Tables S1 and S2, and references within). Only one third of these species have been isolated/observed in adult mosquitoes, reflecting the substantially lower number of fungus sampling efforts in adult mosquitoes compared to collections in immature life stages (Table S2). Two thirds of the isolated fungi belong to the orders Blastocladales (39 species, 38 of which belong to the genus *Coelomomyces*), Eurotiales (28 species, including 16 *Penicillium* sp. and ten *Aspergillus* sp.), Hypocreales (24 species across 14 genera including *Beauveria* and *Metarhizium*), and Saccharomycetales (17 species with more than half belonging to the genus *Candida*). Only one sixth of the isolated water molds are known to be pathogenic, while nearly two thirds of the isolated fungi are either opportunistic, facultative, or obligate pathogens (Table S1). However, these numbers are likely underestimates of the true range of mosquito-fungi interactions. Amplicon-based sequencing of adults from eleven mosquito species identified recently 347 and 1 species of fungi and water molds, respectively. Of these, 96% were described for the first time in or on adult mosquitoes, and their impact on mosquito immunity is entirely unknown.

This section describes the possible encounters with fungi and water molds across the mosquito life history, facilitated by mosquito behavior in their aquatic and terrestrial environments.

### 2.1. Larva-fungus interactions through ingestion

Fungi and water molds in the aquatic environment can enter the mosquito body passively through diverse larval feeding behaviors, including collecting-filtering, as well as grazing on and shredding of decaying and living organic matter (Fish and Carpenter, 1982; Merritt et al., 1992; Yee et al., 2004). Upon uptake, they either are digested, pass through the intestine unharmed, or manage to stay and grow within the mosquito host.

Fungi act as food and provide nutrients for the development of mosquito larvae. An example is the baker's yeast, *Saccharomyces cerevisiae*, that is commonly used for the rearing of mosquito larvae



**Fig. 1. The multitude of potential fungus-mosquito encounters.** Adult mosquitoes are exposed to fungi on a multitude of surfaces during indoor and outdoor resting (A, D, H), blood feeding (C), and sugar feeding (G). Additional fungal encounters can occur during mating (B) and oviposition (E). Mosquito larvae in their aquatic environment are exposed to fungi on plant detritus, within the water column, and at the water surface (F).

(Asahina, 1964). Seven individual yeast species that had been isolated previously from field-collected *Culex theileri* and *Cx. pipiens* larvae, supported the growth of *Cx. pipiens* larvae. This study thus confirmed the nutritional value of yeast ingestion by mosquito larvae (Steyn et al., 2016). Shifts in fungal microbial populations on decaying oak leaf matter in the presence and absence of *Aedes triseriatus* larvae further suggests the ingestion of fungi through larval browsing and grazing (Fish and Carpenter, 1982; Kaufman et al., 2008).

Mosquito feeding behavior also leads to the establishment of commensals in the mosquito hindgut. At least four fungal species in the genus *Smittium* sp., belonging to the order Harpellales, are able to attach and replicate in the hindgut of various mosquito species, with most often no effect on larval development or survival (Lopez-Lastra, 1997; Pereira et al., 2005; Sweeney, 1981; Tuzet and Manier, 1947; White et al., 2006).

During filter feeding and grazing, mosquito larvae do also ingest potential entomopathogenic fungi and water molds. The fungal entomopathogen *Culicinomyces clavissporus* causes infection in *Culex fatigans* larvae only upon ingestion of the fungal conidia (Sweeney, 1975). Likewise, *Smittium morbosum* infects mosquito larvae solely after oral uptake (Sato et al., 1989; Sweeney, 1981). *M. anisopliae* has deleterious effects on the larval hosts after oral uptake, which may be due to subsequent infection of the hemocoel, but at least in certain instances are due to toxicity in the gut (Cheng and Liu, 1990; Butt et al., 2013). The water molds *Leptoglenia chapmanii* and *Lagenidium giganteum* both infect the hemocoel of mosquito larvae after oral uptake of zoospores and by cuticular penetration of the body wall (Zattau and McInnis, 1987; McCray et al., 1973).

## 2.2. Larva-fungus interactions through contact

Entomopathogenic fungi and water molds commonly infect mosquito larvae through active cuticular penetration. Fungi in the genus *Coelomomyces*, including *C. opifexi* are only known to infect mosquito larvae via cuticle penetration (Wong and Pillai, 1980). *C. psorophorae* spores infect only through attachment and cuticular penetration of the head, intersegmental regions, and the base of anal gills (Travland, 1979). In addition to aquatic fungal entomopathogens, soil-borne entomopathogens like *M. anisopliae* and *B. bassiana*, infect larvae of multiple mosquito species via direct penetration of the cuticle. Studies on *M. anisopliae* route of infection in mosquito larvae suggest that infection is mainly due to contact with spores at the water surface, mostly in and around the siphon (Crisan, 1971; Lacey et al., 1988). Likewise, dependent on formulation, *B. bassiana* conidia float on the water surface, and exposure preferentially happens around the perispiracular lobes (Clark et al., 1968).

## 2.3. Transstadial transmission of fungal communities

Transstadial transmission of bacterial microbiota from mosquito larvae to pupae and adults is limited, and has been reported in *Culex tarsalis*, *Cx. pipiens*, *An. gambiae*, and *Ae. aegypti* (Duguma et al., 2015; Moll et al., 2001). Studies conducted on yeast in *Culex* spp. have shown no transmission from larvae to pupae, or to adult stages (Díaz-Nieto et al., 2016; Steyn et al., 2016). These data suggest that transstadial transmission of fungi and water molds, especially if located solely in the midgut, does not occur and is not the source of fungal associations or infections in adult mosquitoes. One exception are the 36 species of *Coelomomyces*. While their persistence to adult mosquitoes occurs at a low frequency, transmission from infected larvae to pupae and adults has been observed repeatedly (Garland and Pillai, 1979; Lucarotti, 1987; Lucarotti and Andreadis, 1995).

## 2.4. Adult-fungus interactions through ingestion

Adult male and female mosquitoes commonly feed on plant nectars

(Foster, 1995). These nectars contain many yeasts that likely are taken up by adult mosquitoes during feeding. The yeast nectar community is diverse and largely depends on inoculation through pollinator species including insects and birds (reviewed in Chappel and Fukami, 2018). Yeast community composition within the guts of pollinators overlaps with the yeast nectar community, strongly suggesting that such composition is largely driven by oral uptake and environmental filtering (Sandhu and Waraich, 1985). Several yeasts that have been identified in field-collected adult mosquitoes, including *C. parapsilosis* and *Hansenula* spp. in *Ae. triseriatus*, as well as *Pichia* spp. in *Aedes japonicus* (Bozic et al., 2017; Muturi et al., 2016; Ricci et al., 2011), overlap with nectar-associated yeasts. Most recently, amplicon-based sequencing of adult *Ae. albopictus* identified a large number of yeast species (Luis et al., 2019). Together, these studies support the notion that nectar-associated yeasts are regularly ingested during nectar feeding by adult mosquitoes (Fig. 1G). Similarly, yeast may be taken up during blood feeding (Fig. 1C). Fungal cultures from human blood samples and blood-fed female mosquitoes share several yeast species, including *C. parapsilosis*, *Rhodotorula* spp., *Saccharomyces* spp., and *Cryptococcus* spp. (Bille et al., 1982; Chang et al., 2001; Muturi et al., 2016).

## 2.5. Adult-fungus interactions through substrate contact

Adult mosquitoes commonly rest in- and outdoors, and thus are exposed continuously to fungi through contact with contaminated surfaces. Mosquitoes, resting in tree cavities or on flower and leaf surfaces, come in contact with a diverse community of microorganisms including yeasts and filamentous fungi (Fig. 1. H, D, Levetin and Dorsey, 2006). Examples of such surface contact exposures is the occurrence of infections of adult *Culex pipiens* with the fungal entomopathogen *Entomophthora conglomerata* and *Entomophthora destruens* in wine cellars and natural caves, respectively (Novak, 1965; Weiser and Batko, 1966). This exposure route is also exploited for vector population control purposes. The provision of *M. anisopliae* and *B. bassiana*-impregnated netting, cloth, and mud panels as indoor resting sites increased the mortality of *An. gambiae* adults (Fig. 1A, Mnyone et al., 2010). Similarly, outdoor mosquito resting sites can be targeted using both entomopathogen species on the surface of clay pot traps, roof covers, and within resting stations like extra-domiciliary odor-baited stations (Luz et al., 2010; Lwetoijera et al., 2010; Mnyone et al., 2010). In addition, transfer of spores through contact with screens impregnated with *B. bassiana* in oviposition traps was used successfully to target female *Ae. aegypti* (Snetelaar et al., 2014).

Gravid female mosquitoes can locate suitable oviposition sites by the detection of secondary metabolites produced by certain fungi (Eneh et al., 2016; Geetha et al., 2003). During oviposition, these females are exposed to these and other soil-borne and/or aquatic fungi through direct contact (Fig. 1E). In how far these brief encounters contribute to fungal-mosquito interactions is currently unclear. The positive correlation of infection prevalence with number of ovipositions suggests that at least *E. conglomerata* may be able to infect *Cx. pipiens* during oviposition (Kupriyanova, 1966). It is feasible that treatment of oviposition sites with fungal entomopathogens to control mosquito egg and early larval stages may also cause infection in adult females (Sousa et al., 2013).

## 2.6. Additional adult-fungus interactions

Mosquitoes can pass on spores that are attached to their own cuticle. Transfer of entomopathogenic fungal spores between individual mosquitoes through mating has been observed in several species. Infection of healthy male *An. gambiae* mosquitoes by *M. anisopliae* was observed post-mating with topically exposed female mosquitoes (Scholte et al., 2004a). Reduction in survival of healthy female *Ae. aegypti* mosquitoes post-mating was observed with both *M. anisopliae* and *B. bassiana*-exposed male mosquitoes, confirming conidial dissemination through



mating in *Ae. aegypti* mosquitoes (Fig. 1B) (García-Munguía et al., 2011; Garza-Hernández et al., 2015; Reyes-Villanueva et al., 2011). As such, mosquitoes can be used to auto-disseminate fungal entomopathogens (Scholte et al., 2004a), and thus extend the reach of these biocontrol agents beyond the initial contact with the impregnated surface (Shah and Pell, 2003). Autodissemination may also extend to oviposition sites (Fig. 1E). A specialized case is the oviposition of resting sporangia of *Coelomomyces* sp., including *C. psorophorae* and *C. stegomyiae* by female mosquitoes. Infected females are sterile, but take blood meals, and actively oviposit sporangia, a behavior that contributes to the dissemination of the fungus to new aquatic environments (Lucarotti, 1987; Laird et al., 1992).

In addition to surfaces, indoor and outdoor air contains a highly diverse fungal spore community (Frohlich-Nowoisky et al., 2009), with some taxa also being found in mosquitoes (Guégan et al., 2018; Muturi et al., 2016). Conidia of *B. bassiana* can be air-borne (Feng et al., 1994), and thus may come in contact with mosquitoes through air rather than surfaces (Clark et al., 1968).

### 3. Getting in: colonization of mosquitoes by fungal and water mold symbionts

Out of the 158 species of fungi for which we found published evidence of isolation from mosquitoes, roughly half can establish longer-term interactions with their hosts. 67 species from six fungal orders are considered pathogens of mosquitoes, using the hemocoel and/or various tissues as growth medium (Section 1, Fig. 2, top and middle panel). Of these, only *Entomophthora* sp. and *Coelomomyces* sp. are obligate pathogens, while the rest are facultative pathogens that switch between pathogenic and saprophytic or even endophytic life styles. In addition, 22 species of fungi in the orders Eurotiales, Hypocreales and Mucorales are opportunistic pathogens, who themselves cannot actively invade the mosquito hemocoel, but can establish an infection if entering through breaches in the cuticle (Fig. 2, top panel). Five *Smittium* species (order Harpellales) are commensals of mosquitoes, attaching to and growing within the hindgut of mosquito larvae (see Section 2.2, Fig. 2, bottom panel).

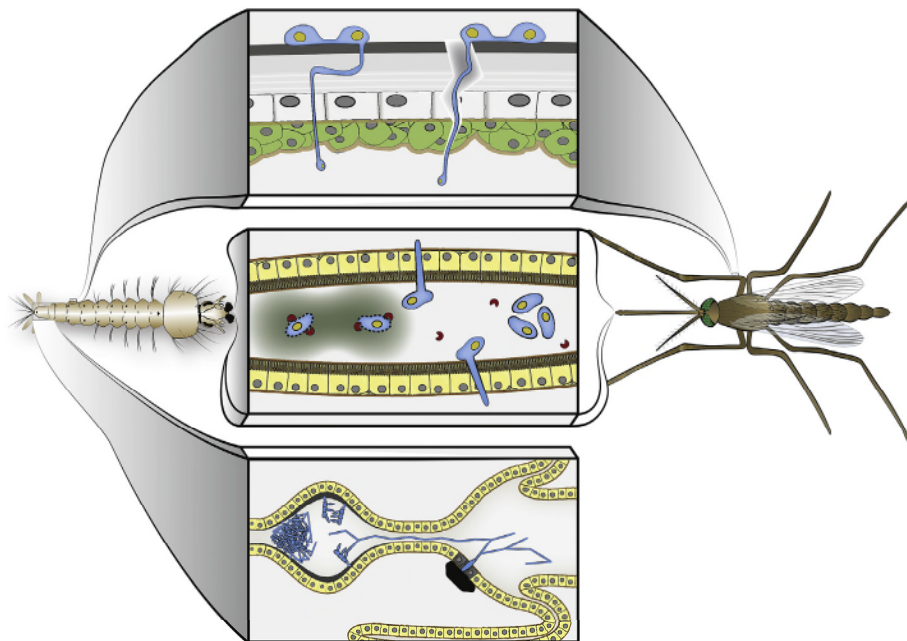
Out of the 43 species of water molds that have been observed and/or isolated from mosquitoes, only five species (in the genera *Lagenidium*, *Leptoglenia* and *Saprolegnia*) are known facultative pathogens of mosquitoes. No commensal water molds have been described in

mosquitoes. This section will briefly describe how some of these symbionts colonize their mosquito hosts, either through breaching the cuticle and/or through ingestion.

#### 3.1. Active penetration of cuticle

As mentioned in Sections 2.2 and 2.5 above, insects often encounter entomopathogens through contact. Upon contact, colonization of the host is achieved through active penetration, a multi-step process that somewhat varies dependent on fungal species (reviewed in Butt et al., 2016; Scholte et al., 2004; Lovett and St. Leger, 2017). In general, active penetration begins with the attachment of spores to the host epicuticle. Fungal spores germinate and form germ tubes that differentiate into specialized attachment organs, called appressoria, that then form a penetration tube, which penetrates the host cuticular layers through pressure exertion and secretion of cuticle-degrading enzymes. Upon reaching the hemolymph, the fungus proliferates as blastospores (yeast-like budded cells) or hyphal bodies (chains of budded cells). As the infection progresses, the insect host succumbs to the infection, either through fungal toxins or by starvation. In most fungal entomopathogens, hyphae break through the cuticle to produce either infective spores or resting structures on the insect cadaver (Fig. 2, top panel; recently reviewed in Lovett and St. Leger, 2017).

Infection by penetration is observed in mosquito larvae and adults, with histological examinations available for several species combinations of fungi/water molds and mosquitoes. Clark et al. (1968) describes the histological manifestation of *B. bassiana* infection in *Cx. pipiens* larvae, observing mycelial growth preferentially around the tracheal trunks, suggesting invasion in and around the trachea. Infection of *Cx. pipiens* larvae by *B. bassiana* led to the disintegration and deformation of the epicuticle, midgut epithelium, and muscles (Benzina et al., 2018). Cuticle penetration has also been commonly observed in mosquito larval infections by *Coelomomyces* sp. (Shoulkamy and Lucarotti, 1998; Wong and Pillai, 1980). *C. psorophorae* zygospore adhesion to *Culiseta inornata* larval cuticle is followed by germination and development of a narrow hyphal body that penetrates the host epidermal cells (Travland, 1979). An ultrastructural study of *C. stegomyiae*-infected *Ae. aegypti* larvae revealed hyphal growth in the muscles with deterioration of myofibrils, degeneration of midgut muscles, and fragmentation of the microvilli that line the malpighian tubules (Shoulkamy et al., 2001).



**Fig. 2. Entry routes of fungal entomopathogens into their mosquito hosts.** Entomopathogens enter mosquito larvae and adults through two distinct routes. Top panel: Fungal and water mold asexual spores attach to the mosquito cuticle and penetrate actively via penetration pegs. Opportunistic fungal pathogens can gain entry through wound sites. Center panel: Ingested spores may be degraded through digestive enzymes in the larval midgut, resulting in toxin release. Some spores may germinate and penetrate through the midgut epithelium, subsequently disseminating throughout the larval body. It is unclear, whether this occurs in adult mosquitoes. Bottom panel: Trichospores of *Smittium* sp. attach to the cuticular lining of the hindgut in mosquito larvae, germinate and grow locally. Hyphae of *Smittium morbosum* can grow anteriorly, and penetrate the posterior midgut epithelium, where they are melanized.

The infection process of water molds is similar. Motile zoospores of *L. giganteum* attach to the cuticle of mosquito larvae, a germ tube penetrates the body wall, hyphae grow in the hemocoel, and start to produce sporangia. The sporangia develop exit tubes that grow out through the cuticle, ultimately releasing zoospore vesicles into the aquatic environment. In addition, resting structures in the form of sexual oospores can also be produced (Fetter-Lasko and Washino, 1983; Brey et al., 1988). Attachment of *L. giganteum* zoospores followed by penetration of the larval cuticle has been observed in *Ae. aegypti*, *An. gambiae*, and *Cx. pipiens* larvae (Brey et al., 1988; Golkar et al., 1993). Similarly, zoospores of *L. chapmanii* have been shown to aggregate, and then enter, via penetration tubes, the head, body, anal papillae, and intersegmental folds of *Ae. aegypti* and *Cx. quinquefasciatus* larvae (Lord and Fukuda, 1988; Zattau and McInnis, 1987).

Exposure of adult *An. stephensi* mosquitoes to *B. bassiana* impregnated filter papers, led to the accumulation and germination of fungal spores on the proboscis, tarsi, legs and wings. During early stages of infection, hyphae invaded tissues and organs, including compound eyes, brain, salivary glands, mouthparts, muscles, midgut, ovaries, and malpighian tubules (Ishii et al., 2017). *Conidiobolus coronatus* infects *Aedes taeniorhynchus* and *Cx. quinquefasciatus* adult mosquitoes through cuticle penetration of the intersegmental interstices, head, and dorsal thoracic regions. Histological studies on infected mosquitoes revealed the dissemination of hyphal bodies in hemocoel, muscles, fat body, and gonads (Lowe et al., 1968; Lowe and Kennel, 1972). Infection of adult *Aedes sierrensis* mosquitoes with *Tolypocladium cylindrosporum*, showed fungal hyphae and hyphal bodies exclusively in the thorax, suggesting active penetration through the cuticle of the thoracic spiracles (Soarés, 1982).

### 3.2. Ingestion of fungal spores

Fungal spore uptake through ingestion has been commonly observed in mosquito larvae. Ingestion of *C. clavosporus*, *B. bassiana*, *M. anisopliae*, and *Aspergillus clavatus* spores was reported for *Aedes*, *Culex*, and *Anopheles* mosquito larvae (Miranpuri and Khachatourians, 1991; Scholte et al., 2004b; Seye et al., 2009). Upon ingestion, the spores may remain in the midgut and release toxins that cause pathology and larval killing (Fig. 2, center panel). Cross sections of *Cx. pipiens* larvae during early stages of infection by *M. anisopliae* revealed the accumulation of intact fungal spores in the gut, confirming spore ingestion. Digestion of fungal spores was observed in cross section of dead larvae where large numbers of spores were disrupted (Crisan, 1971). Ingestion of *M. anisopliae* spores by *Cx. quinquefasciatus* larvae induced larval mortality 24 h post-exposure, suggesting the release of toxins during fungal spore digestion. Guts of infected larvae revealed the accumulation of non-germinated and partially digested fungal spores (Lacey et al., 1988). In either study, oral uptake of *M. anisopliae* did not result in disseminated fungal infection.

Other fungal entomopathogens spread from the midgut lumen throughout the mosquito body by penetration through the midgut epithelium (Fig. 2, center panel). For example, the conidia of *C. clavosporus* adhere to the gut walls of *Cx. fatigans* mosquito larvae, where they germinate and penetrate the gut epithelium (Sweeney, 1975). Ingestion of *B. bassiana* blastospores rather than conidia by *Ae. aegypti* larvae resulted in the spread of fungal spores throughout the larval body 24 h post ingestion, with heavy colonization in the fore-, mid-, and hindgut (Miranpuri and Khachatourians, 1991). Exposure of *Cx. quinquefasciatus* larvae to *A. clavatus* led to the accumulation of fungal spores in the alimentary canal. Infection resulted in the destruction of gut cells and disruption of the gut epithelium in several locations, facilitating fungal dissemination in the mosquito body (Bawin et al., 2016). Ingestion of *Fusarium oxysporum* spores by *Aedes detritus* and *Cx. pipiens* led to the colonization of the gut lumen and penetration of the epithelial lining (Hasan and Vago, 1972).

Several species in the genus *Smittium* establish symbiotic

relationships due to attachment to the cuticular lining of the hindgut (Fig. 2, bottom panel; Lichtwardt, 1996). In most cases, the symbiotic relationship remains commensal, and the fungus is shed with every molt. Pathology can be induced by hyphal overgrowth causing complete blockage of the alimentary canal, as is observed for *Smittium culisetae* (Williams and Lichtwardt, 1972). The only species within this genus that can be considered a facultative pathogen of mosquito larvae is *Smittium morbosum* (Coluzzi, 1966; Sweeney, 1981; Sato et al., 1989). Upon initial attachment to the hindgut, *S. morbosum* grows anteriorly, and hyphae penetrate the posterior midgut epithelium. Systemic infection is prevented by the mosquito immune system through strong melanization at the basal side of the midgut epithelium. However, this melanotic mass anchors the hyphae, and either allows transstadial transmission, or leads to incomplete molting, causing larval death.

In addition to infection by cuticle penetration, as described in section 3.1, zoospores of the water mold *L. chapmanii* have been shown to be ingested by *Ae. aegypti* larvae (Pelizza et al., 2008; Zattau and McInnis, 1987). Upon ingestion, the zoospores germinated in the midgut, elongated and formed branched hyphae that penetrated the peritrophic matrix and midgut epithelium, reaching the hemocoel. Once in the hemocoel, infection proceeded similarly to infection initiated by cuticle penetration (Zattau and McInnis, 1987).

To the best of our knowledge, there is currently no evidence that adult mosquitoes commonly ingest entomopathogenic fungi, or that oral uptake of fungal entomopathogens results in adult mosquito infection.

### 3.3. Fungal entry through injury

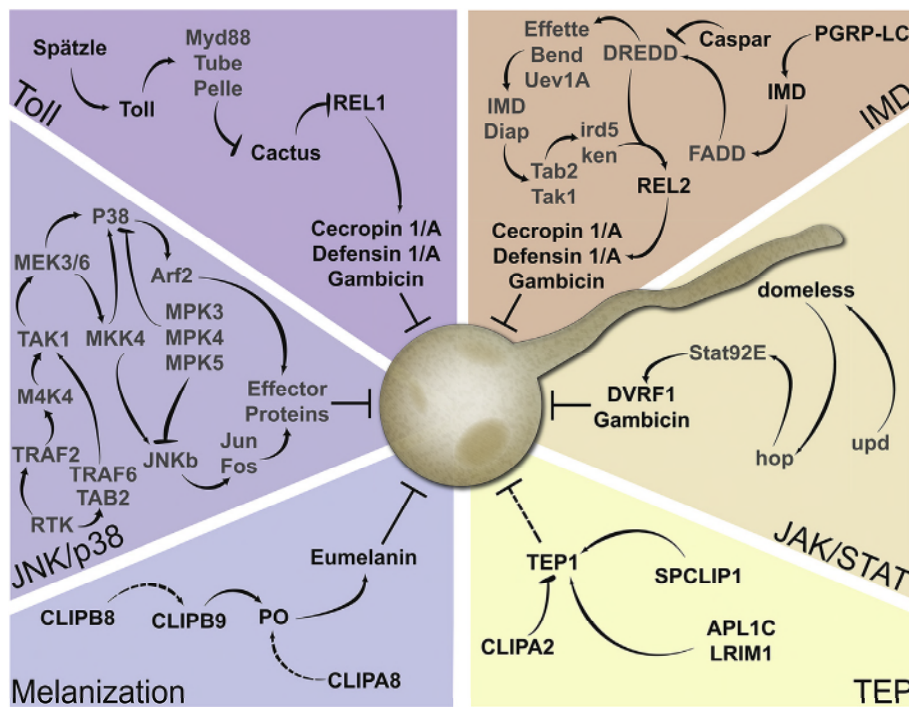
Fungal infection of mosquitoes can be facilitated through injuries that breach the cuticle, as these allow entry of opportunistic pathogens (Fig. 2, top panel). The entomopathogenic fungus *Pythium sp.* cannot infect mosquito larvae through penetration, but infects and kills mechanically injured *Aedes*, *Anopheles*, and *Culex* mosquito larvae (Clark et al., 1966). Experimental infections can also be achieved through intrathoracic conidial spore injection, as observed for *B. bassiana* in adult *An. gambiae* mosquitoes and *M. anisopliae* and *A. clavatus* in *Cx. quinquefasciatus* larvae (Bawin et al., 2016; Yassine et al., 2012). While this route of infection perhaps occurs less frequently in the field and is difficult to exploit for biological control, it can facilitate research of molecular mosquito-fungal interactions and antifungal immunity.

## 4. Antifungal immune mechanisms in mosquitoes

Once a water mold or a fungus has penetrated the mosquito cuticle and entered the hemocoel, it encounters both efficient cellular and humoral immune responses. Survival thus depends on its ability to largely avoid or eventually overcome these immune responses. Nevertheless, immune escape is incomplete. For example, *B. bassiana* is recognized, and its growth is limited by the mosquito immune system. The outcome of infection is thus strongly influenced by the immune competency of the host. Somewhat surprisingly, few data are available to date on the molecular interactions of mosquitoes with fungi. This section will provide a brief overview of the molecules and pathways currently known to be involved in fungus recognition and killing in various mosquito species.

### 4.1. Recognition

$\beta$ -1,3-glucans are the known molecules on the surface of fungal cell walls that are recognized by insect immune systems (Lu and St. Leger, 2016). Studies in various insects have identified  $\beta$ -1,3-glucan recognition proteins (GRPs) and Gram-negative binding proteins (GNBPs) bind to  $\beta$ -1,3-glucan and trigger downstream immune responses, including Toll pathway activation and/or melanization (Jiang et al., 2004; Matskevich et al., 2010; Ochiai and Ashida, 2000). The only molecule



**Fig. 3.** Immune modules that regulate mosquito antifungal immunity. Fungi that penetrate epithelia and reach the hemolymph, as indicated by the germinating blastospore drawing, are attacked by multiple antifungal molecules. These molecules can be expression products of signal transduction pathways, including the Toll, IMD, JAK/STAT and MAPK pathways. Alternatively, these molecules are proteolytic activation products of humoral killing modules, circulating in the hemolymph, including PO and TEP1, which are products of the melanization and complement-like pathways, respectively. Proteins are bolded if their role in mosquito antifungal immunity is supported experimentally. Other key factors of the mosquito immune modules are listed in gray.

currently confirmed to bind  $\beta$ -1,3-glucan in mosquitoes is a GRP from *Armigeres subalbatus* (AsGRP). AsGRP binds to curdlan, an insoluble  $\beta$ -1,3-glucan polymer, and is required for melanization of some bacterial species (Wang et al., 2006, 2005). If AsGRP contributes to melanization of fungal species is unclear. GGBP2 was found to be transcriptionally upregulated in *An. gambiae* mosquitoes that were challenged by injection of dead *B. bassiana* conidia (Aguilar et al., 2005). Whether GGBP2 can directly bind to fungal surfaces, however, remains to be tested. The water mold *L. chapmanii* is also detected in mosquito larvae, as evidenced by localized immune responses to its surface (Zattau and McInnis, 1987). The cell wall of *L. chapmanii*, and that of all water molds, does not contain chitin, and it is unclear how and which molecules on its surface are responsible for immune recognition.

Thioester-containing proteins (TEPs) bind to many foreign surfaces and are critical for pathogen recognition and innate immunity in mosquitoes (Blandin and Levashina, 2004). The activation of the complement-like pathway in mosquitoes leads to the deposition of thioester-containing protein 1 (TEP1) on foreign surfaces (Fig. 3). TEP1 acts as an opsonin and promotes the phagocytosis of bacteria (Levashina et al., 2001; Moita et al., 2005) and lysis of the rodent malaria parasite, *Plasmodium berghei* (Blandin et al., 2004). Several studies point to the involvement of TEPs in antifungal immunity in mosquitoes. Infection of *Ae. aegypti* mosquitoes with *B. bassiana* and *Cordyceps javanica* resulted in upregulation of TEP22 in the fat body and midgut of infected mosquitoes (Ramirez et al., 2018a, 2019). Knockdown of TEP22 increased the susceptibility of *Ae. aegypti* to *B. bassiana* infection, confirming the role of TEP22 in antifungal immune response (Wang et al., 2015). In *An. gambiae*, TEP1 binds to *B. bassiana* hyphal bodies (Yassine et al., 2012). Knockdown of TEP1 decreased hyphal melanization and increased susceptibility of *An. gambiae* mosquitoes to infection by *B. bassiana* (Yassine et al., 2012). Together, these studies suggest that TEPs are important molecules for recognition of fungi in the mosquito hemocoel and critically contribute to the antifungal immune response in mosquitoes. However, the binding partner of TEPs on the surface of fungi, or any other surfaces, awaits identification.

In addition to  $\beta$ -1,3-glucan, other sugar moieties on the fungal surface may be recognized. Neuraminic acid and/or sialic acid on the surface of *B. bassiana* blastospores are potential binding partners for lectins (Wanchoo et al., 2009). It remains to be seen whether mosquito-

derived lectins can recognize these sugars and contribute to antifungal immunity or immunity against water molds.

#### 4.2. Killing mechanisms

##### 4.2.1. Cellular immune responses

Cellular immune responses, mediated by hemocytes, can kill fungal cells within the hemolymph in insects. Hemocytes phagocytize, encapsulate, and nodulate blastospores and hyphal bodies (Lavine and Strand, 2002). Phagocytosis is initiated by the recognition and binding of phagocytic cells to fungal particles, leading to particle engulfment through cytoskeleton modification and intracellular vesicular transport to the phagosome (Jiravanichpaisal et al., 2006). Phagocytosis of blastospores of the fungal species *M. anisopliae*, *B. bassiana*, *Cordyceps farinosa*, *Metarhizium rileyi*, *Cordyceps fumosorosea*, and *Candida albicans* has been observed in several insect species (Hung et al., 1993; Hung and Boucias, 1992; Kawakami, 1965; Ouedraogo et al., 2003; Pendland and Boucias, 1996). In mosquitoes, granulocytes and, less commonly, prohemocytes exhibit phagocytic activity (Hillyer and Strand, 2014). Inoculation of *An. albimanus* with *S. cerevisiae* resulted in the melanization of yeast cells and encapsulation by plasmatocytes. However, phagocytosis of yeast cells was not observed (Hernández-Martínez et al., 2002).

Cellular immune responses have also been reported in response to water mold infections. *L. giganteum* increased transiently total hemocyte numbers in *Cx. quinquefasciatus* larvae, as well as the number of granulocytes compared to other hemocyte cell types (Fei and Huai-en, 2001). This suggests that *L. giganteum* can stimulate cellular immunity in mosquitoes. Indeed, hemocytes in *Ae. aegypti* larvae form loose capsules on *L. giganteum* hyphae. In how far these cellular responses limit *L. giganteum* infection of mosquito larvae remains to be tested.

##### 4.2.2. Antimicrobial peptides (AMPs)

In addition to cellular immune responses, insects mount humoral immune responses against fungi, including the expression of antimicrobial peptides (AMPs, Fig. 3). Most insect AMPs are short amphipathic peptides that are active against gram-positive and/or gram-negative bacteria (Boman, 2003; Tonk and Vilcinskis, 2017). All mosquito genomes annotated to date encode members of the defensin, cecropin,



dipterecin, and gambicin AMP families (Bartholomay et al., 2010; Christophides et al., 2002; García Gil de Muñoz et al., 2008; Neafsey et al., 2015; Waterhouse et al., 2007). In addition, a putative *attacin* gene has been identified in *Ae. aegypti* and *An. gambiae* (Waterhouse et al., 2007). Of these, defensin A and C (DEF1), cecropin A (CEC1), and gambicin (GAM1) are currently implicated in antifungal immunity. Infection of *Cx. quinquefasciatus* larvae with *Metarhizium brunneum* conidia and blastospores upregulates *DEF1*, *CEC1*, and *GAM1* (Alkhaibari et al., 2018). Infection of adult *Ae. aegypti* with either *B. bassiana* or *C. javanica* upregulated *defensin A* and *C*, as well as *cecropin D* and *G* (Ramirez et al., 2019). Synthetic *An. gambiae* CEC1 exhibited antifungal activity against the yeasts *S. cerevisiae*, *Cryptococcus neoformans*, and *C. albicans*, and filamentous fungi belonging to *Aspergillus* and *Fusarium* genera (DeLucca et al., 1997; Vizioli et al., 2000). Similarly, CEC1 purified from *Ae. aegypti* hemolymph exhibited antifungal activity against *C. albicans*, *C. neoformans*, *S. cerevisiae*, *Fusarium culmorum*, and *Neurospora crassa* (Lamberty et al., 2001; Lowenberger et al., 1999). DEF, isolated from female *An. gambiae* midgut tissues, inhibited hyphal growth of the filamentous fungi *Botrytis cinerea*, *F. oxysporum*, *F. culmorum*, and *N. crassa* (Vizioli et al., 2001b). GAM1, purified from *An. gambiae* 4a-3B cell-conditioned media, inhibited hyphal growth of *N. crassa* (Vizioli et al., 2001a). Beyond known AMPs, few studies have specifically searched for antifungal peptides (AFPs) in insects (Al Souhail et al., 2016; Faruck et al., 2016), and none have been conducted in mosquitoes.

#### 4.2.3. Melanization

In addition to AMP expression, the arthropod-specific melanization immune response is commonly observed against fungi (Fig. 3). Aromatic amino acids are converted to eumelanin, which is deposited on surfaces that are recognized by the immune system as foreign. This acellular encapsulation is thought to kill pathogens through site-directed cytotoxicity and/or by blocking nutrient uptake (Nappi and Christensen, 2005; Nappi and Vass, 1993). A key enzyme in melanization is phenoloxidase (PPO), which is expressed from nine to ten paralogous genes in all mosquito genomes annotated to date (Bartholomay et al., 2010; Neafsey et al., 2015; Waterhouse et al., 2007). Infection of *An. gambiae* larvae with *L. giganteum* triggered a heavy melanization response of hyphae 2 h post-infection, while *Ae. aegypti* larvae had a lower melanization response of less than 10% of hyphae (Golkar et al., 1993). Melanization in *Ae. aegypti* larvae was observed at the entry points of *L. chapmani* zoospores and along the hyphal growth into the coelomic cavity (Zattau and McInnis, 1987). Yeast cells, when deposited into the hemocoel of mosquitoes, trigger an efficient melanization response. *S. cerevisiae*, injected into the hemocoel of *An. albimanus*, is unable to grow and establish an infection in the hemolymph, due to the melanization of the yeast cells (Hernández-Martínez et al., 2002). Likewise, *C. albicans* is efficiently melanized and killed in the hemolymph of *Cx. quinquefasciatus* (Da Silva et al., 2000). Melanization in response to fungal entomopathogen infection has been observed in larvae of multiple mosquito species. *Anopheles amicus*, *Anopheles annulipes*, *Cx. fatigans*, and *Ae. tarsalis* larvae form melanotic capsules around *Culicinomyces* at all sites of infection (Sweeney, 1975). The fungus is encapsulated on the cuticle of the larval foregut and hindgut, the fungal penetration sites between gut and hemolymph, and inside the hemolymph. Cuticular pigmentation and darkening of the head capsule was observed in early larval instars of *An. stephensi* when exposed to *B. bassiana* (Prasad and Veerwal, 2010). The melanization response in *An. gambiae* confers partial resistance to *B. bassiana* infection, as fungal load and mosquito death rate increased when the melanization response was experimentally decreased (Yassine et al., 2012). The resistance to fungal infection was, in part, mediated by TEPI, as its depletion from mosquito hemolymph resulted in less deposition of phenoloxidase on the surface of *B. bassiana* hyphal bodies. This study thus provided first molecular insight into mosquito antifungal immunity beyond AFPs, providing additional evidence that TEPI

functions as an opsonin for melanization (Yassine et al., 2012).

#### 4.3. Regulation of antifungal immunity through signal transduction pathways

Several signal transduction pathways contribute to mosquito immunity, including Toll, IMD, JAK-STAT, JNK, and p38 signal transduction pathways (Fig. 3, Bian et al., 2005; Frolet et al., 2006; Meister et al., 2009, 2005; Shin et al., 2005). Below we briefly summarize current knowledge of these signal transduction pathways with regards to their (i) activation by fungal infections, (ii) control of antifungal molecule expression, and (iii) impact on fungal infection outcome.

##### 4.3.1. Toll pathway

The Toll pathway is a major immune signaling pathway in insects, whose role in antifungal immunity is well documented (Lemaître et al., 1996). Named after its transmembrane receptor Toll, it consists of an extracellular protease cascade that activates the Toll ligand Spätzle, and an intracellular signal transduction cascade that translocates an NF- $\kappa$ B transcription factor into the nucleus (Hoffmann and Reichhart, 2002; Lemaître, 2004; Rhodes and Michel, 2017; Valanne et al., 2011). In mosquitoes, the NF- $\kappa$ B transcription factor downstream of the Toll pathway is REL1 (Barillas-Mury et al., 1996; Meister et al., 2005; Shin et al., 2005). *B. bassiana* infection activates the Toll pathway in *Ae. aegypti* and *An. gambiae* adult mosquitoes (Dong et al., 2012; Rhodes et al., 2018; Shin et al., 2006). *C. javanica* and *Beauveria brongniartii* also activate the Toll pathway in *Ae. aegypti* adult mosquitoes (Ramirez et al., 2018a). Manipulation of the pathway in mosquitoes strongly influences fungal infection outcome. In *Ae. aegypti*, knockdown of *Toll5A* and *Spz1C* decreased survival in *B. bassiana*-infected mosquitoes (Shin et al., 2006). Similarly, *Rel1* knockdown in *Ae. aegypti* and *An. gambiae* mosquitoes decreased mosquito survival to infection by *B. bassiana* (Bian et al., 2005; Rhodes et al., 2018; Shin et al., 2005). Knockdown of *Cactus*, a negative regulator of the Toll pathway, increased survival of *An. gambiae* mosquitoes to infections with *B. bassiana* (Rhodes et al., 2018). The Toll pathway regulates the expression of several known AMPs with antifungal activity, including *DEF1*, *CEC1*, and *GAM1* (Barillas-Mury et al., 1996; Garver et al., 2009; Luna et al., 2006; Shin et al., 2005; Zhang et al., 2017). In addition, the Toll pathway regulates the basal expression levels of *TEPI* (Frolet et al., 2006). As the Toll pathway controls about 10% of the mosquito transcriptome, its influence on fungal infection likely goes beyond the regulation of these few antifungal factors.

##### 4.3.2. IMD pathway

The IMD pathway is a major immune signal transduction pathway in the gut and regulates malaria parasite killing in mosquitoes (Antonova et al., 2009; Chen et al., 2012; Garver et al., 2009, 2012; Meister et al., 2005, 2009). Its downstream NF- $\kappa$ B transcription factor in mosquitoes is called REL2 (Fig. 3, Antonova et al., 2009; Meister et al., 2005). Infection of *Ae. aegypti* mosquitoes with *B. bassiana*, *B. brongniartii*, and *C. javanica* upregulated *Rel2* transcript levels in the midgut and fat body six days post-infection (Ramirez et al., 2018a, 2019), suggesting the activation of the IMD pathway upon fungal challenge.

The IMD pathway confers resistance to fungal infections in mosquitoes. Knockdown of *Rel2* in *B. bassiana* and *C. javanica*-infected *Ae. aegypti* adult mosquitoes decreased mosquito survival to infection and increased fungal load in infected mosquitoes (Ramirez et al., 2018b). Similarly to the Toll pathway, the IMD pathway regulates the expression of *DEF1*, *CEC1*, and *GAM1* (Antonova et al., 2009; Garver et al., 2009; Luna et al., 2006; Meister et al., 2005, 2009; Ramirez et al., 2018b; Zhang et al., 2017). Altered expression of these antifungal molecules may contribute to the antifungal resistance that is exerted through the IMD pathway.



#### 4.3.3. JAK-STAT pathway

In mosquitoes, the JAK-STAT pathway is involved in antiviral and anti-*Plasmodium* immunity (Carissimo et al., 2015; Gupta et al., 2009; Souza-Neto et al., 2009). The pathway is activated by ligand-binding to the domeless (Dome) receptor, and ultimately translocates the transcription factor Stat92E to the nucleus (Fig. 3, Zeidler and Bausek, 2013). Infection of adult *Ae. aegypti* with *B. bassiana* upregulated the expression of *Dome* and the STAT-regulated anti-dengue restriction factor 1 (*DVRF1*). Similarly, the expression of *STAT* was upregulated in *Ae. aegypti* mosquitoes infected by *B. bassiana*, *Be. brongniartii*, and *Cordyceps amoenerosea* (Ramirez et al., 2018b, 2018c). Knockdown of *Dome* or *DVRF1* decreased mosquito survival to *B. bassiana* infection (Dong et al., 2012). In *Ae. aegypti* cells, the JAK-STAT pathway upregulates *GAM1* expression in response to *C. albicans* exposure (Zhang et al., 2017), which may contribute to JAK-STAT pathway-mediated antifungal immunity in mosquitoes.

#### 4.3.4. MAPK pathways

Less is known about the role of the JNK and p38 MAP-kinase signaling pathways in mosquito immunity, including responses to fungal infection. In *Ae. aegypti* mosquitoes, infection with *C. albicans* upregulates MAP kinase kinase 4 (MAPK4), which is upstream to JNK and p38 kinases (Wu and Cho, 2014). Likewise, infection with *B. brongniartii* upregulates the expression of *JNK* in the midgut and fat body (Ramirez et al., 2018a). It is unclear whether either of these pathways contribute to antifungal immunity in mosquitoes. However, in *Drosophila*, p38 mutant flies exhibited increased susceptibility to infection by *B. bassiana* and *Aspergillus fumigatus* (Chen et al., 2010).

#### 4.4. Regulation of antifungal immunity through protease cascades

In addition to transcriptional regulation, several mosquito humoral immune responses are regulated through proteolytic activation of key immune factors and enzymes, including TEP1 and PPO (Nakhleh et al., 2017; Rhodes and Michel, 2017). Proteolytic activity is regulated through cascades of Clip-domain containing serine proteinases (CLIPs) and their proteolytically inactive homologs (clip-SPHs, most often CLIPAs). TEP1 function is augmented by two clip-SPHs, SPCLIP1 and CLIPA2. SPCLIP1 is essential for TEP1 deposition on the surface of microbes and acts as a positive regulator of the complement-like pathway (Povelones et al., 2013). CLIPA2 acts as a negative regulator through inhibiting the production of active TEP1 during infection (Yassine et al., 2014). Knockdown of *CLIPA2* decreased susceptibility of *An. gambiae* to *B. bassiana* in a TEP1-dependent manner, confirming the role of TEP1 in antifungal immunity (Kamareddine et al., 2016).

*An. gambiae* CLIPA8 is a positive regulator of phenoloxidase activation and required for melanization in response to *P. berghei* parasites, bacteria, and *B. bassiana* (Schnitger et al., 2007; Yassine et al., 2012). Knockdown of *CLIPA8* abolished the melanization of *B. bassiana* hyphae, and decreased resistance to *B. bassiana* infection (Yassine et al., 2012). While several CLIPs are required for PO activation in mosquitoes (An et al., 2011; Zhang et al., 2016), their role in antifungal immunity in mosquitoes is untested.

### 5. Summary and outlook

The mosquito immune system has evolved in the context of continuous encounters between mosquitoes and fungi ranging from food to foes. Many of these encounters indeed are beneficial to the mosquito. Yeast hold nutritional value for mosquito larvae (Asahina, 1964; Steyn et al., 2016), and may generate a hypoxic environment in the larval midgut required for mosquito development (Valzania et al., 2018). Encounters with potential opportunistic fungal pathogens may remain benign for the mosquito due to physical and physiological barriers, including the cuticle and digestive enzymes (Gillespie et al., 2000). However, a significant number of fungal entomopathogens can

overcome barriers to infection and colonize the mosquito hemocoel with often lethal consequences to the host. It is thus not surprising to find all branches of the mosquito immune system to be engaged in antifungal immunity. The current body of literature describes some of these interactions on a histological, and to a much lesser extent, molecular level.

Important knowledge gaps remain. (i) Out of the many potential mosquito-fungal encounters, little is known of the mosquito immune system's role in fungal interactions beyond defense against a small number of well-studied entomopathogens in adult infections. Comparative studies using multiple mosquito and fungal species combinations would be valuable to address whether a core of anti-fungal immunity mechanisms are engaged across mosquito species, and what biotic and abiotic factors influence the efficacy of these immune responses. Mosquito larval immunity is largely unexplored, and its contribution to antifungal defense is unclear. It is likely that exposure of the larval immune system impacts adult immunity. This is important to explore, as it could impact vector-borne pathogen susceptibility and transmission rates.

- (ii) The molecular identity of antifungal molecules in both mosquito larvae and adults is largely unexplored. Naturally occurring AFPs constitute a numerous and structurally highly diverse group of peptides (van der Weerden et al., 2013), and it is likely that mosquitoes produce antifungals beyond canonical AMPs. Beyond knowledge gain, mosquito AFPs could have practical applications, including their use as biomarkers of exposure, and natural product fungicides (Rautenbach et al., 2016; van der Weerden et al., 2013).
- (iii) During colonization, entomopathogenic fungi clearly interact with mosquito epithelia, including the epidermis, tracheal linings, and gut epithelium. However, the role of epithelial immunity in limiting fungal colonization is unknown. Mosquito epithelial immune responses contribute to maintenance of a healthy microbiota and defense against vector-borne disease pathogens, and likely also limit fungal infections through similar mechanisms.
- (iv) In laboratory studies, boosting mosquito basal immunity reduces fungal entomopathogen infections (Rhodes et al., 2018; Yassine et al., 2012). Different strains and species of fungal entomopathogens differ in their virulence against the same mosquito species (Blanford et al., 2012). Entomopathogen virulence is also influenced by abiotic factors, such as temperature (Heinig et al., 2015). However, in contrast to field and laboratory studies on variation in host susceptibility to vector-borne pathogens (Collins et al., 1986; Gubler, 1979; Gubler and Rosen, 1976; Huff, 1929, 1927; Niaré et al., 2002; Wallis et al., 1985), mosquito host genotype influence on fungal entomopathogen infection is, to our knowledge, unexplored. Such studies may help to identify non-canonical antifungal immune responses in mosquitoes. Such knowledge could further aid biocontrol programs to select for strains that even more readily overcome mosquito immunity.
- (v) Our knowledge of mosquito-fungal interactions is mostly confined to entomopathogens, due to their potential for biological control of mosquitoes. In contrast to bacterial microbiomes, investigations of naturally occurring fungal associations with mosquito larvae and adults are just beginning. So far, a handful of studies have identified yeasts and filamentous fungi associated with mosquitoes. Future studies should explore whether the transient nature of these associations impact mosquito physiology and shape the immune status of mosquitoes.

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## Appendix A. Supplementary data

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